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The Effects of Diazepam on Brain 5-HT and 5-HIAA in Stressed and Unstressed Rats

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COPLAND, A. M. AND D. J. K. BALFOUR. *The effects of diazepam on brain 5-HT and 5-HIAA in stressed and unstressed rats.* PHARMACOL BIOCHEM BEHAV 27(4) 619–624, 1987.—Diazepam, administered to rats at a high dose (25 mg/kg PO) has been shown to have no effect on the plasma corticosterone response to the stress of an elevated open platform. It did however, reduce the plasma corticosterone in rats repeatedly exposed to the apparatus. Diazepam-withdrawal from stress-habituated rats increased plasma corticosterone ($p<0.01$) whereas withdrawal of diazepam from unstressed rats had no effect on plasma corticosterone. It is concluded that this effect of diazepam-withdrawal may reflect the development of dependence upon the drug. Significant effects were not observed following the administration of a lower non-selective dose (5 mg/kg PO) of diazepam and, therefore, it is not clear whether dependence to its sedative, rather than the anxiolytic properties have been measured. Acute diazepam (25 mg/kg) increased ($p<0.05$) hippocampal 5-hydroxyindoleacetic acid; its withdrawal from unstressed rats after 40 days reduced ($p<0.01$) hypothalamic 5-hydroxytryptamine. There was no evidence that the effects of diazepam or its withdrawal on plasma corticosterone in stressed rats were associated directly with changes in brain 5-hydroxyindoles.

Diazepam Diazepam-withdrawal 5-Hydroxytryptamine 5-Hydroxyindoleacetic acid Hippocampus Hypothalamus Cerebral cortex Plasma corticosterone Stress Rats

IT is now generally accepted that benzodiazepines exert a majority of their effects by stimulating receptors on the $GABA_A$ receptor complex and thereby facilitating the responses to GABA at these receptors [9,17]. However, it is becoming increasingly clear that some non-benzodiazepine drugs, such as buspirone, can also exert anxiolytic effects and that these effects do not appear to depend upon enhanced stimulation of $GABA_A$ receptors [27]. There is evidence to suggest that the anxiolytic properties of buspirone may depend upon its ability to interact with serotonergic pathways in the brain [12,16] although the subject remains controversial [30]. There are reports that benzodiazepine anxiolytic drugs also interact with brain 5-hydroxytryptamine (5-HT) systems. For example *in vitro* studies have shown that diazepam stimulates the release of ^{3}H-5-HT from slices of rat midbrain preincubated with ^{3}H-5-HT whereas it inhibits release from slices of cerebral cortex [8]. Studies in this laboratory have shown that diazepam stimulates the spontaneous release of ^{3}H-5-HT release from hippocampal synaptosomes whereas it inhibits the potassium-evoked release of the monoamine [1]. There is also evidence that benzodiazepines alter the turnover of 5-HT in rodent brain *in vivo* [26,29] although the possibility that these changes mediate the anxiolytic properties of the drugs has been questioned [14,22]. One of the objectives of the study reported in this paper was to investigate the possible role of brain 5-HT systems in the effects of diazepam on the plasma corticosterone response to an elevated open platform, a form of stress in which its effects had not previously been examined.

There is now good evidence to suggest that chronic treatment with benzodiazepines is often associated with the development of dependence upon the drugs [25]. Balfour [2] has discussed the possibility that environmental factors may be a significant determinant in the extent to which dependence to these drugs develops and, in particular, that dependence may be related to their effects on the processes by which animals habituate to aversive environments. Benwell and Balfour [6] have reported results which implicated hippocampal 5-HT systems in the process by which rats habituate to the aversive properties of an elevated open platform. This investigation, therefore, also examined the possibility that diazepam-dependence may be associated with regionally-selective changes in the concentrations of 5-HT or its principal metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the hippocampus.

METHOD

Animals

The animals used were male Sprague-Dawley rats, bred in the Animal Services Unit, Ninewells Hospital, from stock purchased from Charles River (UK) Ltd. They weighed approximately 120–150 g at the beginning of the studies and, when not in the experimental room, were allowed free access to food and water. The animals were housed in groups of three. The animal house lights were on between 0800 and 2000 hr daily.

TABLE 1

EFFECTS OF DIAZEPAM AND ACUTE EXPOSURE TO THE ELEVATED OPEN PLATFORMS ON PLASMA CORTICOSTERONE

Treatment	Plasma Corticosterone (μg/100 ml) Home Cage	Elevated Platforms
Vehicle	10.6±0.6	25.0±2.7
Diazepam (5 mg/kg)	8.9±1.8	25.9±3.6
Diazepam (25 mg/kg)	9.0±0.6	31.5±3.2

The results are means±SEM of 6 observations. The plasma corticosterone levels measured in the rats tested on the elevated platforms were significantly higher, $F(1,30)=44$, $p<0.001$, than those measured in the rats that remained in their home cages.

TABLE 2

EFFECTS OF CHRONIC DIAZEPAM AND ITS WITHDRAWAL ON PLASMA CORTICOSTERONE IN UNSTRESSED RATS AND RATS EXPOSED REPEATEDLY TO THE ELEVATED PLATFORMS

Treatment	N	Plasma Corticosterone (μg/100 ml) Home Cage	Elevated Platforms
Vehicle	10	8.6 ± 1.2	17.1 ± 1.5*
Diazepam (5 mg/kg)	5	7.4 ± 1.6	13.5 ± 1.8
Diazepam-withdrawn	5	7.4 ± 1.6	18.7 ± 2.6*
Diazepam (25 mg/kg)	10	10.8 ± 2.7	8.8 ± 1.6†
Diazepam-withdrawn	10	11.1 ± 2.4	28.0 ± 3.2*†

The animals were treated for 40 days, the rats exposed to the elevated platforms being tested for 30 minutes starting 30 minutes after each dose of vehicle or diazepam. The results are mean ± SEM. *Significantly different from rats which remained in their home cages ($p<0.01$); †Significantly different from rats treated chronically with the vehicle ($p<0.01$); and tested in the same environment. N=Number of rats tested.

Acute Studies

The animals were first habituated to the handling and dosing procedure to be used by transferring them daily to the experimental room and administering a small volume of water (1 ml/kg) via an orogastric tube. On the day of the experiment the animals were divided at random into three groups (N=12 per group) and given diazepam (5 mg/kg or 25 mg/kg) or the vehicle (40% propylene glycol in water) orogastrically. Thirty minutes after the injections, half the rats in each group were stressed by being placed individually on to an elevated open platform [9] for 30 minutes. The remaining animals were left in their home cages. Sixty minutes after the injections, and, for the stressed rats, immediately upon removal from the aversive environment, the rats were killed by cervical dislocation and the brain and a blood sample taken for biochemical analysis.

Chronic Studies

Groups of rats were given diazepam (5 mg/kg or 25 mg/kg) or the vehicle orogastrically for 40 days. Thirty minutes after each injection, 10 of the rats treated with the vehicle or 5 mg/kg diazepam and 20 of the rats given 25 mg/kg diazepam were placed in the aversive environment for 30 minutes. On day 40 half the rats previously treated with diazepam (5 mg/kg or 25 mg/kg) were given vehicle in place of the drug suspension in order to examine the effects of diazepam-withdrawal. The remaining rats in each treatment group (N=8 for vehicle-treated controls; N=16 for each of the diazepam-treated groups) were left in their home cages after the injections. On day 40 diazepam was withdrawn from half the rats in each of the diazepam-treated groups and replaced by vehicle alone. All the rats were killed 60 minutes after receiving the last dose of diazepam or vehicle on day 40 and brain and blood samples taken for biochemical analysis as before.

In order to examine temporal relationships between the effects of diazepam and its withdrawal on responses to the aversive environment and habituation to the environment, further groups of animals (N=4 per group) were killed after 10 or 20 daily sessions on the platforms.

Biochemical Analysis

The plasma samples were stored overnight at −20°C prior to analysis. The brain tissue was stored at −70°C for 2–4 weeks prior to dissection and analysis. Plasma corticosterone was assayed using the procedure of Mattingly [23] adapted for small plasma volumes. The concentrations of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were measured using the method of Reinhard *et al.* [27].

Statistical Analysis

The data were analysed using a two-way ANOVA with drug treatment and exposure to the aversive environment as independent factors. The effects of the number of test sessions on the responses to the aversive environment were analysed using a two-way ANOVA with drug treatment and duration of treatment as the independent factors. Post hoc analysis was performed using Newman-Keuls test. Linear correlation coefficients were determined using the method of least squares.

RESULTS

Acute Studies

Acute exposure to the elevated platforms caused a significant increase, F platforms $(1,30)=44$, $p<0.001$, in the plasma corticosterone concentration (Table 1). Neither of the doses of diazepam tested had any significant effects on the plasma corticosterone levels measured in either the unstressed rats or on the raised levels observed in the rats exposed to the elevated platforms. In a separate experiment, group of rats (N=5 per group) were pretreated with vehicle or diazepam (5 or 25 mg/kg) for 6 days prior to being tested acutely on the elevated platforms. The plasma corticosterone levels in these rats (28±4 μg/100 ml for the rats given 5 mg/kg diazepam, 32±3 μg/100 ml for the rats given 25 mg/kg diazepam) were also not significantly different from those measured in vehicle-treated rats tested on the platforms (24±5 μg/100 ml). Acute diazepam exerted a significant ef-

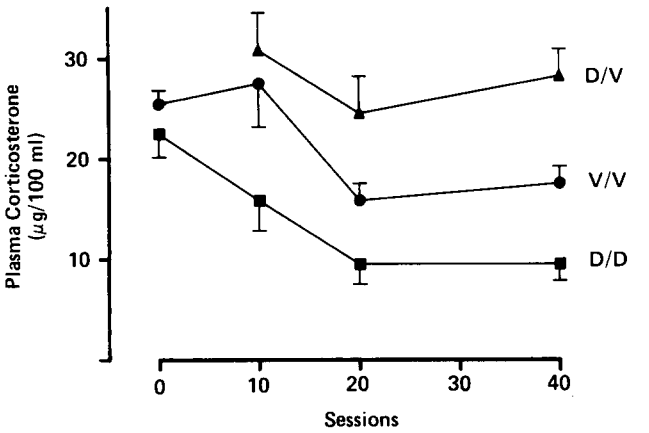

FIG. 1. Plasma corticosterone levels were measured in rats tested on the elevated platforms after each injection of vehicle (V/V) or 25 mg/kg diazepam (D/D) and in diazepam-withdrawn rats (D/V). The results are means±SEM of 6 observations (Session 1), 4 observations (Sessions 10 and 20) or 10 observations (Session 40).

fect, F treatment (2,30)=4.09, $p<0.05$, on the concentration of 5-HIAA in the hippocampus. Post hoc analysis revealed that the acute administration of the higher dose of diazepam increased the ($p<0.05$) 5-HIAA concentration in the hippocampus from 0.335±0.030 (vehicle-treated rats) to 0.433±0.045 μg/g, whereas the lower dose of the drug had no significant effect. No other significant changes in response to diazepam were observed and acute exposure to the elevated platforms was also found to be without effect on the 5-hydroxyindole concentrations measured.

Chronic Studies

Analysis of the measurements made in rats treated for 40 days showed that the plasma corticosterone levels in the rats placed on the elevated platforms remained significantly higher, F platforms (1,70)=38, $p<0.001$, than those measured in the rats which were left in their home cages (Table 2). The treatment the rats received also exerted a significant effect, F treatment (4,70)=9.14, $p<0.001$, and the interaction between the effects of exposure to the platforms and the effects of treatment, F platforms by treatment (4,70)=4.55, $p<0.01$, was significant. Post hoc analysis showed that, in the unstressed rats, neither diazepam administration nor its withdrawal had any significant effects on the plasma corticosterone concentration. In the vehicle-treated rats the plasma corticosterone level was increased significantly ($p<0.01$) in the rats tested on the elevated platforms. This increase was attenuated by the administration of diazepam, significantly so ($p<0.01$) when the higher dose of the drug was given. Diazepam-withdrawal from the rats tested on the platforms caused an increase in plasma corticosterone which was significant ($p<0.01$) for the rats given 25 mg/kg diazepam.

Exposure to the platforms for 40 days caused a significant increase, F platforms (1,70)=9.57, $p<0.01$, in the concentration of 5-HT in the cerebral cortex (from 0.157±0.013 μg/g to 0.243±0.027 μg/g). The administration of diazepam had no effects on cerebrocortical 5-HT and the interaction with the effect of exposure to the platforms was also not significant. Although the effects of exposure to the platforms and of treatment with diazepam on hypothalamic 5-HT were not

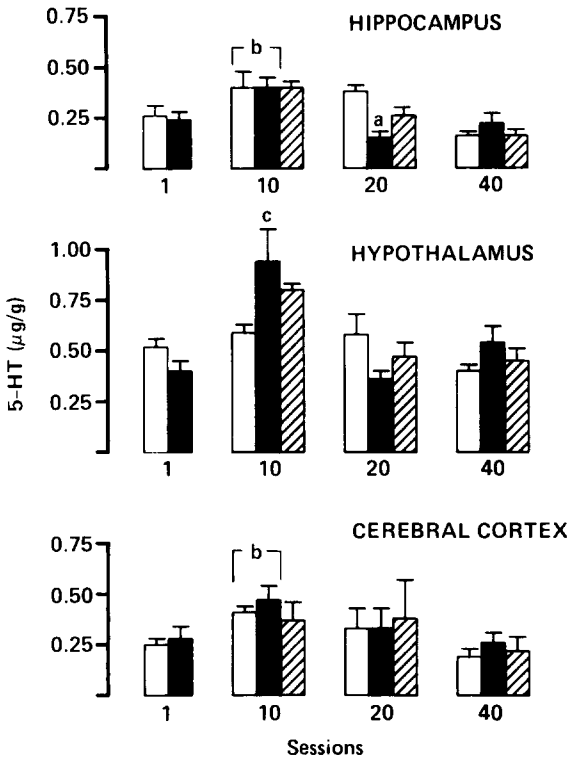

FIG. 2. The 5-HT concentrations were measured in rats given the vehicle (clear bar) or 25 mg/kg diazepam (solid bar) prior to each session on the elevated platforms and diazepam-withdrawn rats (striped bar) tested on the platforms. The results are means±SEM of 6 observations (Session 1), 4 observations (Session 10 and 20) or 10 observations (Session 40). a: significantly different from vehicle-treated rats tested for the same number of sessions ($p<0.01$); significantly different from levels measured after session 1: (b=$p<0.05$; c=$p<0.01$).

significant, the interaction between the two effects did achieve statistical significance, F platforms by drug-treatment (4,70)=3.79, $p<0.01$. Post hoc analysis showed that the withdrawal of the higher dose of diazepam from the unstressed rats caused a significant reduction ($p<0.01$) in hypothalamic 5-HT from 0.493±0.039 μg/g to 0.324±0.035 μg/g which was not observed in the rats tested on the open platforms. The 5-HT concentration in these unstressed diazepam-withdrawn rats was significantly lower ($p<0.05$) than that measured in the withdrawn rats (0.0469±0.066 μg/g) exposed to the platforms for 40 days. Neither chronic exposure to the platforms nor treatment with diazepam had any significant effects on hippocampal 5-HT. No significant changes in the concentrations of 5-HIAA were observed for any of the brain regions examined.

Exposure to the platforms for 10 sessions was not associated with any significant changes in the plasma corticosterone concentration when compared with increase after session 1 (Fig. 1) and the reduction in plasma corticosterone observed in the rats treated with diazepam (25 mg/kg) also failed to achieve significance. However after 10 sessions on the platforms the concentrations of 5-HT in hippocampus, F sessions (1,18)=4.43, $p<0.05$, hypothalamus, F sessions (1,18)=10.3, $p<0.01$, and cerebral cortex, F sessions (1,18)=6.05, $p<0.05$, were significantly higher than those measured after only one session (Fig. 2). In the hypothala-

TABLE 3

LINEAR CORRELATIONS WITH PLASMA CORTICOSTERONE MEASURED IN RATS TESTED REPEATEDLY ON THE OPEN PLATFORMS

	Treatment Group		
	Vehicle	Diazepam	Diazepam-Withdrawn
Hippocampal 5-HT	0.65†	0.54*	−0.19
Hypothalamic 5-HT	0.13	0.50*	−0.27
Cerebrocortical 5-HT	0.21	0.10	−0.56*
Hippocampal 5-HIAA	0.00	0.20	0.34
Hypothalamic 5-HIAA	−0.27	0.02	−0.44
Cerebrocortical 5-HIAA	−0.23	−0.02	−0.53*

*$p<0.05$, †$p<0.01$.
N=18 per group.

mus the interaction between sessions and drug-treatment was also significant, F sessions by treatment (1,18)=7.97, $p<0.01$, and post hoc analysis showed that, for this area of the brain, the increase was only significant ($p<0.01$) for the rats given diazepam.

Analysis of the results for sessions 10 to 40 indicated that both the number of sessions, F sessions (2,44)=6.0, $p<0.01$, and treatment, F treatment (2,44)=31, $p<0.001$, exerted significant effects on plasma corticosterone. Post hoc analysis showed the plasma corticosterone concentration was lower after session 40 than after session 10 for both the vehicle- and diazepam-treated rats ($p<0.01$ and $p<0.05$ respectively) but that this was not the case for the diazepam-withdrawn rats. Habituation to the platforms between sessions 10 and 40 was also associated with significant reductions in the concentrations of 5-HT in the hippocampus, F sessions (2,47)=26, $p<0.001$, hypothalamus, F sessions (2,47)=13.3, $p<0.001$, and cerebral cortex, F sessions (2,44)=4.16, $p<0.05$. In the hippocampus the interaction between the effect of sessions and drug-treatment was also significant, F sessions by treatment (4,43)=3.35, $p<0.05$, and post hoc analysis revealed that, after 20 test sessions, diazepam caused a significant reduction in hippocampal 5-HT ($p<0.01$) when compared with the vehicle-treated rats. No significant effects on the concentrations of 5-HIAA in any of the brain regions examined were observed.

In the vehicle-treated rats repeatedly tested in the elevated platforms (sessions 10 to 40), hippocampal 5-HT correlated significantly ($p<0.01$), although not particularly closely, with the plasma corticosterone concentration (Table 3). This correlation was also significant ($p<0.05$) for the diazepam-treated rats. In these rats the correlation with hypothalamic 5-HT was also significant ($p<0.05$). In the diazepam-withdrawn rats, plasma corticosterone exhibited significant negative correlations with cerebrocortical 5-HT and 5-HIAA.

In order to examine the possibility that the acute administration of diazepam (25 mg/kg) to vehicle-treated rats which had habituated to the elevated platforms might exert the same effects on plasma corticosterone as diazepam-withdrawal, a further group of 4 vehicle-treated rats were exposed to the elevated platforms for 19 days. Thirty minutes prior to session 20, the rats were given diazepam (25 mg/kg) and blood samples taken at the end of the session. The plasma corticosterone levels measured in these rats (13±2 μg/100 ml) were not significantly different to those measured in the diazepam and vehicle-treated rats which received their habitual treatment prior to session 20 but were significantly less ($p<0.01$) than those measured in diazepam-withdrawn rats tested on the platforms.

DISCUSSION

This study has shown that, although acute exposure to the stress of the elevated platform had no significant effects on brain 5-HT and 5-HIAA levels, repeated exposure to the platform was associated with increased levels of 5-HT which, in the cerebral cortex, remained significantly elevated in rats tested in the apparatus for 40 sessions. Studies in other laboratories have shown that exposure to aversive stimuli increases the biosynthesis and release of 5-HT in the brain [11, 18, 19] and therefore it seems reasonable to suggest that the increased 5-HT levels, observed in the present investigation may be the result of repeated stress-induced stimulation of 5-HT release. This conclusion is supported by the fact that the greatest increases in 5-HT were measured in rats which had been exposed to the platforms for 10 sessions and that the increases were less marked in rats which had habituated to the procedure.

The experiments have shown that the high dose of diazepam tested also exerted effects on brain 5-HT although there was no evidence to suggest that the changes were associated with the effects on the plasma corticosterone response to the aversive environment. Indeed the reduction in hypothalamic 5-HT evoked by diazepam-withdrawal only occurred in the unstressed rats. Lister and File [21] have proposed that the increased 5-HT levels observed in the chlordiazepoxide-treated rats are associated with its sedative rather than its anxiolytic properties. The results reported in this paper are consistent with this hypothesis since they have shown that diazepam only increased hippocampal 5-HIAA when given acutely at a high dose which has previously been shown to be sedative [3]. In the rats repeatedly tested on the platform diazepam decreased hippocampal 5-HT on day 20. This effect could be related to the reduction in the plasma corticosterone measured in these rats although this seems unlikely since no reduction in hippocampal 5-HT was observed after session 40 when the drug also reduced the plasma corticosterone response to the apparatus.

It was perhaps surprising to find that the lower dose of diazepam used in this investigation did not attenuate the increase in plasma corticosterone evoked by exposure to the elevated platform since similar doses of the compound have been found to reduce the plasma corticosterone response to a number of different anxiogenic stimuli [4, 5, 7, 20]. However, studies by Barlow *et al.* [5] have suggested that some forms of stress are too severe for benzodiazepines to exert a measurable effect on the corticosterone levels. It seems reasonable to suggest, therefore, that a failure to observe an effect of diazepam in rats tested acutely on the platforms could reflect the severity of the test procedure and that the effect of the drug only becomes apparent when the aversive properties of the apparatus are diminished by habituation.

In many patients the abrupt cessation of treatment with benzodiazepine anxiolytic drugs is reported to cause withdrawal effects which often include feelings of severe anxiety and panic [25]. In the present study diazepam-withdrawal was shown to increase plasma corticosterone in rats tested on the elevated platforms but to have no effects in unstressed

rats. The data, therefore, appear consistent with the hypothesis that diazepam can interact with the process by which rats habituate to an aversive environment and that its withdrawal from the rats tested on the platforms was stressful. This effect of diazepam-withdrawal could be the result of state-dependent learning. However, this seems unlikely since reversing the protocol by giving diazepam to rats which had habituated to the apparatus without the drug did not evoke a similar increase in plasma corticosterone. Thus it seems reasonable to suggest that the study has provided some evidence that diazepam dependence may be associated with its effects on habituation to aversive environmental stimuli and that exposure to such stimuli may be an important factor in the expression of the withdrawal effects.

Studies in other laboratories have suggested that the development of dependence upon benzodiazepines may be associated with a reduction in the sensitivity of $GABA_A$ receptors including, specifically, those located on 5-HT-secreting neurones [10,15]. The experiments reported in this paper have provided no direct evidence to suggest that the 5-HT systems in the brain regions examined are implicated in the effects of diazepam-withdrawal although the extent of the withdrawal effect did appear to correlate negatively, and only to a limited extent, with reduced 5-HT turnover on the cerebral cortex. Significant withdrawal effects were only observed for the rats given the higher dose of diazepam. It is possible, therefore, that these studies have measured dependence upon the sedative rather than the anxiolytic properties of the drug. Indeed there is evidence to suggest that rats develop tolerance to the effects of low doses of benzodiazepines on plasma corticosterone [4,13]. Nevertheless further studies with different aversive environments are necessary to clarify the relative roles of the anxiolytic and sedative properties of these drugs in the development of dependence and to establish any relationship between the severity of the aversive stimulus used and its potential to provoke the development of dependence upon these drugs.

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